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KLEBSIELLA OCCURRENCE, SIGNIFICANCE AND DETECTION IN WATER SYSTEMS: A PROGRESS REPORT

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16. ABSTRACT

Frequent occurrence of Klebsiella in coliform colonization problems found in water supply distribution has prompted the development of a new medium (m-Kleb agar) for specific detection. The medium has excellent differential characteristics, and an average 94% Klebsiella recovery rate when used in the membrane filter procedure. This media may also be used as a streak plate to verify Klebsiella occurrence in the MF verification procedure or as a supplementary test in the multiple tube confirmation process.

A proposed action plan with two levels of response to coliform occurrences in public water supplies is discussed. The purpose would be to separate operation corrective measures to be performed under task force guidance from more serious conditions requiring a boil water notification to the public.

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Many of the water systems reporting coliform occurrences in their distribution networks have noted that the predominant organism was a Klebsiella strain. Speciation most frequently reveals these strains are Klebsiella pneumoniae, but several other species (K. rhinoscleromatis, K. oxytoca and K. ozaenae) have also been reported on occasion (1-7). While these organisms can be effectively controlled by adequate disinfection, there can be organism protection afforded by particulate material, porous pipe sediments, macroinvertebrates, and disinfectant demand products in the water (8-12). Furthermore, encapsulation of these coliforms can occur in hostile water environments (13), triggered perhaps by phosphates and depletion levels of iron and magnesium salts. Formulation of a capsular, slime coating around Klebsiella cells provides a measure of protection from the effects of chlorine and other disinfectants and the organisms adjust their metabolism to survival on minimal nutrients adsorbed by pipe sediments. Once such organisms become established in this environment, growth beyond meager persistence can result in the periodic sloughing of cells into the flowing water. This condition can persist until either hydraulic shearing effects limit colony growth or contact with elevated disinfectant residuals penetrates the protective habitat and inactivates the microbial population.

Klebsiella colonization in the distribution network has created much discussion and controversy on the interpretation of the public health significance of these members of the total coliform group in water systems. There appears to be no supporting evidence currently available to suggest that bacterial pathogens would also persist in a similar environmental niche in the distribution system nor has the problem been properly investigated. Significance, interpretation and an appropriate plan of action are important in this situation because the public water system may be in violation of the Federal Drinking Water Regulations related to coliform occurrences.

Klebsiella significance

Faced with this problem, it is essential that the state-of-knowledge on Klebsiella in the water environment be reviewed to evaluate their public health significance. Origins of some Klebsiella may be the feces of warm blooded animals. Approximately 30-40 percent of humans carry this coliform in their intestinal tract (14,15) and the Klebsiella density may range from 10^6 to 10^8 organisms per gram of feces. Approximately 60 to 85% of all Klebsiella from feces and clinical specimens are positive in the fecal coliform

test (16). K. pneumoniae is the cause of 18% of all urinary tract infections, occasional bacteremia, cholecystitis and meningitis and may be a major bacterial infection of respiratory, gastrointestinal, EENT areas associated with whirlpool bath exposures (17-20). This species also accounts for 4% of bacterial pneumonias, being most common in people whose health is compromised by illness and injury (21,22).

Other Klebsiella have their origins in the environment, being found on fruit and vegetable produce, dairy products, seed embryos, internal and external tree tissues, hay and cotton. Wood pulp, paper mills, textile finishing plants and sugar cane processing operations may release 10^4 to 10^6 klebsiellae per ml of effluent and comprise about 50 to 90 percent of the total coliform population (33-39). Fresh redwood lumber used to construct water storage tanks have been shown to be a source of Klebsiella that contaminate water transmitted in the distribution network (31,40-43).

Surface and unprotected ground water receive both environmental and fecal sources of Klebsiella. Environmental strains are introduced to the source of supply from urban and rural runoff plus the discharges from wood, paper and textile processing wastes while fecal derived Klebsiella enter in the point source discharges from municipal sewages, meat processing works and non-point source discharges from feedlots and farm pastures (35,44-47).

From the available information, the public health significance of Klebsiella can be very difficult to interpret unless supplemented by information provided on several key growth responses and antibiotic resistance characteristics. Fortunately, the biochemical characteristics of commercial multi-test systems will provide speciation for most isolates purified from coliform colonies on M-Endo agar cultures. However, this identification procedure is costly and takes additional time once coliforms have been detected on M-Endo medium. What is more desirable is the availability of a differential medium with excellent selectivity that correlates with speciation via the multi-test system. In addition, the candidate medium must also provide high recovery rates of Klebsiella in natural waters. This identification must be supplemented with a simple verification procedure that separates fecal strain from environmental organism.

Detection

Several Klebsiella media have been proposed including MacConkey-Inositol-Carbenicillin agar (MCIC) (48), m-K medium (49), m-FCIC (47), acriflavine-Violet Red Bile agar (AVRB) (22), and Double Violet agar (DV) (50). Inspection of these media formulations reveals a variety of agents were used to selectively differentiate Klebsiella from other organisms, including carbenicillin, various bile salts, uric acid, inositol, adonitol, and acriflavine. Differential indicators incorporated in these membrane filter media used different combinations or individual constituents, such as phenol red, violet red, methyl violet 2B and crystal violet to achieve optimum color contrast and detect differential reactions. Evaluation of four of these media (MCIC, m-K medium, AVRB, and DV) on a variety of ambient waters, indicated, of the group, MCIC agar provided the best selective recovery of Klebsiella, because of the relative ease of target organism identification in polluted waters. What can be disturbing in the proposed use of MCIC agar in drinking water analysis,

is the conflicting reports of either 66% or 95% recovery for Klebsiella present in various natural waters (48,51). Projecting the lower average recovery rate to contaminated drinking water, where coliform densities are often few in numbers, some Klebsiella could go undetected. Furthermore, reported verification of only 75% (51) would not provide adequate reliance on data interpretation for a rapid operational response.

Since coliform occurrences in water must be detected at a level of one organism per 100 ml, our research investigation was initiated to develop an improved Klebsiella medium that would be applicable to special monitoring in water systems. As a second objective, the candidate medium would be tested in parallel with MCIC and M-K agar for potential improvement in detecting Klebsiella from polluted waters. Finally, efforts would be made to develop a testing protocol for determining the origin of Klebsiella that could be useful in deciding an appropriate level of action needed by water authorities.

METHODS AND DEVELOPMENT

Medium Development

In designing a new Klebsiella medium (m-Kleb), attention was given to optimizing performance in conjunction with a membrane filter procedure because 100 ml or more sample volumes of drinking water would be examined. To simplify medium preparation, Phenol Red agar base (Difco) was selected to which carbenicillin and adonitol were added as selective agents for Klebsiella separation from other coliforms. Sodium lauryl sulfate was included to suppress a significant portion of other organisms in the water flora. Past experience in development of M-FC broth (52) suggested the use of aniline blue, in addition to phenol red in the agar base formulation, to provide appropriate differential contrast in detecting the pH shift resulting from fermentation of adonitol. During early experimentation with this medium it became evident that Klebsiella colonies were often very large (6-8 mm in diameter) and might become confluent on the MF surface at densities of 60 colonies or more. This undesirable condition was corrected by the inclusion of 2 ml ethanol per liter after sterilization. Ethyl alcohol (95%) and carbenicillin must be added after sterilization, otherwise both ingredients are destroyed with exposure to 121°C for 15 minutes. Medium formulation with Phenol Red agar base is given as version A in Table 1, or prepared from individual ingredients as version B.

a) medium preparation

To prepare m-Kleb agar, add 31.0 grams of Phenol Red agar base, 5.0 grams of adonitol, 0.1 gram of aniline blue and 0.1 gm sodium lauryl sulfate in 1000 ml of distilled water. The mixture is sterilized by autoclaving for 12-15 minutes at 121°C. After autoclaving, the agar preparation is cooled to 50°C in a water bath, then 20 ml of 95% ethyl alcohol (not denatured) and 0.05 g carbenicillin are added per liter of medium. The mixture is thoroughly dispersed and the medium then dispensed into 50 x 9 mm plastic culture plates. Prepared culture medium can be held for use over a 20 day period, provided storage is at 4°C to prevent a decrease in the potency of carbenicillin.

b) sample processing

Following sample filtration, the membrane with entrapped bacteria is carefully placed over the m-Kleb agar surface, avoiding the entrapment of air between the agar-membrane filter interface. Cultures are incubated for 24 hours at 35°C. Klebsiella colonies are deep blue to blue-gray in color, while other colonies are most often pink or occasional pale yellow.

c) pure culture performance

Pure cultures of both recent coliform and non coliform isolates were used to test differential characteristics and recovery. Each pure culture was identified by the API-20E system and held on either tryptic soy agar or brain heart infusion agar with frequent transfers. Test cultures used to evaluate medium performance were 18-24 hour transfers. Sensitivity of m-Kleb agar to detection of long term persisting Klebsiella was also investigated. Washed Klebsiella cells were placed in sterile Super Q (Millipore Corp.) laboratory pure water and held at room temperature for 20 days. Plating on the experimental medium (m-Kleb) and plate count agar were used to measure recovery of persisting Klebsiella. The relative recovery or sensitivity to persisting Klebsiella was expressed as a ratio calculated by dividing the recovery on m-Kleb medium by that of the reference medium (PCA).

d) media comparisons

Evaluation of m-Kleb medium against MCIC and m-K agars was performed on natural samples to compare selectivity, sensitivity and reliability of differentiation. Both alternative agar preparations were prepared, as described by the authors. The water samples were further characterized for total and fecal coliform bacteria using m-Endo and M-FC media, as described in Standard Methods (53). Standard plate count agar (PCA) was used in either a pour plate or spread plate technique to determine the heterotrophic bacterial density in the natural samples and the resulting counts were related to suppression on the selective media.

e) colony verification

Following incubation on m-Kleb or MCIC agar media for 24 hours or after 48 hours on m-K agar, colony counts were made and colony verification initiated to test medium reliability for differentiation of target organisms. A minimum of 10 typical colonies per culture or 10% of similar colonies from high density samples were selected at random for further processing. Atypical colonies were selected at a rate of 5 colonies per culture for the verification process. Each selected colony was purified by streaking on plate count agar with incubation for 24 hours at 35°C. From each purification plate, an isolated colony was selected and inoculated into the API-20E multi test system for species identification.

RESULTS AND DISCUSSIONS

Pure culture performance

Results of the m-Kleb agar performance with pure cultures to determine specificity and sensitivity of the medium are shown in

Table 2. Klebsiella strains were consistently dark blue or blue-gray on m-Kleb medium and the recovery rate generally equalled or exceeded that observed on plate count agar used as a base line. This average Klebsiella recovery rate far exceeded similar studies obtained in the evaluation of four other media (52). In that media comparison study, the average recoveries for Klebsiella pure cultures ranged from 52 to 69 percent. Most non Klebsiella pure culture organisms that grew on m-Kleb were pink, with the exception of one strain each of Enterobacter aerogenes and Enter. agglomerans which produced a blue color. A Yersinia enterocolitica strain, one Aeromonas hydrophila and one K. oxytoca produced yellow colonies.

Recovery of persisting Klebsiella strains was also given consideration in medium performance, because surviving coliforms have minimal nutrient support in drinking water, and may also be stressed by exposure to disinfection. Furthermore, it has been suggested that stressed coliforms have poor recovery in any medium containing a bile product as part of the formulation (54). Data in Table 3 was developed from a 20 day survival of Klebsiella in laboratory water (Millipore Super Q system), and shows that recovery on m-Kleb agar (which contains sodium lauryl sulfate) was equal to that obtained from plate count agar. The regrowth of Klebsiella in this laboratory water may have been a result of organics passing through an exhausted carbon filter section of the Super Q system that had not been changed in many months. It was also interesting to note that the Klebsiella strain developed a mucoid mutant that became more dominant with storage time.

Natural Water Samples

While it would have been desirable to evaluate the different Klebsiella media on actual samples of water from water supply distribution networks, this was not possible because of the unpredictability of obtaining samples which contained coliforms. Therefore, media evaluation was performed on a variety of natural water samples collected from a spring, lake, creeks, river and a sewage system.

The results from the examination of natural waters and wastewater for Klebsiella on three Klebsiella media are shown in Table 4. Data for the experimental medium, m-Kleb, also included verified Klebsiella densities calculated from the percent of Klebsiella colonies identified to species by the API-20E system. Verified m-Kleb counts were also compared to counts obtained on MCIC and m-K agars and the ratios indicate m-Kleb medium to be equal to or slightly better in recovery of Klebsiella. This may, in some respects, be a reflection of the improved differential color contrasts developed by the m-Kleb agar indicator system on the membrane filter compared to the indicators used in the two other media. It was also noted that there was a significant reduction in the density of non-Klebsiella colonies on m-Kleb agar and often these colonies appeared to be smaller in diameter than those observed in parallel tests with the other two media.

Inspection of the data for Klebsiella and fecal coliforms in these natural waters indicates Klebsiella are often in higher densities than fecal coliforms. This observation supports the argument, that more environmental strains of Klebsiella are to be found in

natural waters and sewage effluents than fecal coliforms, and that fecal Klebsiella are less numerous than the environmental strains of the same genus.

Verification of random colonies on m-Kleb cultures of natural waters was done with the API 20-E system to relate colony color with specific species of bacteria. Data in Table 5 demonstrates the high degree of accuracy achieved in the differential characteristics of m-Kleb agar (94.6% positive correlation of blue colonies with Klebsiella species). Only three strains of the Enterobacter genus gave a false positive (blue color) reaction and few species of other coliforms were found to grow; those few being pink in color. E. coli, the major fecal coliform was only found on two isolations from all water and waste sources and both strains produced pink colonies, suggesting the excellent selectivity of the m-Kleb agar formulation.

m-Kleb Agar as A Screening Procedure

The demonstrated excellent differential characteristics of m-Kleb agar supports its application as a Klebsiella screening procedure for both the MF and MPN total coliform methods. When sheen colonies are found on membrane filter cultures from drinking water, verification of gas production is essential to demonstrate these colonies are total coliforms (53). This test is done by simultaneous inoculation of lactose broth and brilliant green lactose broth tubes. Extending the verification process on drinking water samples to include inoculation in EC broth for 24 hrs at 44.5°C to determine if the coliform is fecal and a streak plate prepared on m-Kleb agar (incubated at 35°C for 24 hr) would provide additional information on the status of the sheen colony, i.e.: a) it is a total coliform, b) it is or is not a fecal coliform, and c) it is or is not a Klebsiella (Fig. 1.).

Klebsiella strains which are positive in the fecal coliform test are of greatest concern, because their presences suggests fecal contamination is involved which could also contain some intestinal pathogens. Of equal concern are Klebsiella strains with antibiotic resistant factors (16,55-57). These R-factor strains suggest they may have been released in wastes from the hospital environment, in feedlot runoff or acquired this resistance factor through gene transfers with other organisms in water treatment process basins. Since Klebsiella can be an opportunistic pathogen, those strains that have multiple resistance might be a serious health problem to some susceptible consumers.

In those laboratories that use the multiple tube test, confirmation for Klebsiella could also be accomplished by streaking positive lactose cultures onto plates of m-Kleb agar. Isolated blue colonies detected in 24 hrs after incubation at 35°C would be presumptive evidence that the total coliform population included or consisted solely of, Klebsiella.

Klebsiella Significance in Water Systems

With realization that Klebsiella strains are found in two widely different origins, i.e., fecal contamination and environmental surroundings, there is concern about their public health significance. Careful attention to water treatment processes does provide effective

barriers to coliform releases into the water distribution network. Conversely, ineffective control of turbidity, inadequate disinfection, poor operation and maintenance practices in other treatment processes and the distribution network will provide opportunities for the passage and colonization of coliforms.

Passage of any coliforms into the plant effluent indicates barriers are ineffective and there is increased chance for waterborne pathogens to follow the same pathway. The coliform occurrence escalates to an immediate need to issue a "boil water" order if any of these occurrences in finished water contain fecal coliforms.

Projecting the same logic to the distribution system, no coliforms should be found in a properly maintained pipe network that also includes reservoirs and standpipes. To consistently achieve this goal requires careful control on all water treatment operations, plus adequate sanitizing procedures for all new line extensions and pipe repairs in addition to periodic cleaning of storage reservoirs and standpipes. With the inclusion of an effective cross connection control program and a thorough flushing of the distribution system on a yearly basis, chlorine demand would be low, coliform colonization improbable and chlorine residual concentration required, minimal. Areas of perpetual concern will, however, always remain around building construction sites where there is ground disturbance and with the deterioration of pipe sections in service for over 75 years.

Response to Coliform Occurrences

Coliform colonization of the distribution system is of questionable sanitary significance. What needs to be determined is whether such occurrences are a new incursion of contamination or a reoccurrence of coliforms that have become colonized in the distribution system. While there is no magic test to determine the status of a coliform occurrence, several patterns fit the two different situations. Coliform incursions are often limited to one area of the distribution system during the first hours of contamination, and can be accompanied with increased heterotrophic bacterial counts and a loss of disinfectant residual. At this time, coliform monitoring of plant effluent may reveal a few coliforms only if larger samples (liter size) are examined, or only if composited sampling is done during a 24 hour period, to detect a pulse of coliform organisms related to treatment adjustments. If these occurrences are a result of a back flow from low line pressure, fecal coliforms and a turbidity rise may also occur briefly.

Coliform colonization and regrowth in the distribution system fits a different pattern (58-60). Speciation of the coliforms reveals these organisms belong to the genera Klebsiella, Enterobacter or Citrobacter. Most frequently, Klebsiella species predominate and a free chlorine residual ranging from 0.1 to 2 mg/l will also be present. These occurrences may relate either to warm water periods of a seasonal nature or to major changes in water pH adjustments to reduce a corrosion problem. It is not surprising to note coliform densities ranging from 1 to 125 organisms per 100 ml, and find their occurrence to be widespread in the distribution network. Investigation of past records often shows this problem to be a repeat of past summers, with positive samples ranging form 4 to 11% of all bacteriological samples. However, the percent occurrence and monthly average

may be depressed by intensifying the number of samples examined.

Since coliform colonization in the distribution system may be more an indication of excessive sediment accumulation, rising chlorine demand and increased slime formation, the need for public notification takes on a different aspect. For these reasons, there is a need to develop two levels of response: the first level of action would be formation of a task force (utility, State, and Federal water authorities) to review coliform colonization problems, recommend corrective action and meet at frequent intervals (weekly or daily, if necessary) to review progress made by the water plant operations to correct the deficiency. The second level of action would be initiated immediately if any of the following factors were involved: no followup on task force recommendations; waterborne outbreak occurs; a loss of disinfection treatment; or a fecal coliform occurrence is identified. The overall objective is to formulate a responsive action plan rather than drive the monthly coliform average into a false sense of compliance by increased sample analyses. The other concern is one of public over-reaction from frequent boil water orders that serves little purpose other than motivate some consumers to resort to water supply alternatives, some of which may have even less desirable water qualities than public water supplies.

SUMMARY

Frequent occurrence of Klebsiella in coliform colonization problems found in water supply distribution has prompted the development of a new medium (m-Kleb agar) for specific detection. The medium has excellent differential characteristics, and an average 94% Klebsiella recovery rate when used in the membrane filter procedure. This media may also be used as a streak plate to verify Klebsiella occurrence in the MF verification procedure or as a supplementary test in the multiple tube confirmation process.

A proposed action plan with two levels of response to coliform occurrences in public water supplies is discussed. The purpose would be to separate operation corrective measures to be performed under task force guidance from more serious conditions requiring a boil water notification to the public.

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TABLE 1. m-KLEB MEDIUM FORMULATION

VERSION A	
PHENOL RED AGAR BASE (DIFCO)	31 G.
ADONITOL	5 G.
SODIUM LAURYL SULFATE	0.1 G.
ANILINE BLUE	0.1 G.
DISTILLED WATER	1000 mL.
VERSION B	
PROTEOSE PEPTONE #3	10 G.
SODIUM CHLORIDE	5 G.
ADONITOL	5 G.
BEEF EXTRACT	1 G.
SODIUM LAURYL SULFATE	0.1 G.
ANILINE BLUE	0.1 G.
PHENOL RED	0.025 G.
AGAR	15 G.
DISTILLED WATER	1000 mL.

AUTOCLAVE EITHER VERSION FOR 12-15 MINUTES AT 121°C. COOL TO 50°C THEN ADD 20 ML OF ETHANOL (NOT DENATURED) AND 0.05 G. CARBENICILLIN PER EACH LITER OF MEDIUM. FINAL pH 7.4 $\pm$ 0.2. DISPENSE INTO CULTURE PLATES.

TABLE 2. *Klebsiella* Recovery Study on m-Kleb Agar

Organism	Fecal Coliform Reaction	Trial Run	Plate Count Agar* Pour	Plate Count Agar* Spread	m-Kleb*	Ratio m-Kleb/PCA Spread
K. pneumoniae	+	1	38	29	37	1.3
		2	23	32	30	0.9
		3	25	21	21	1.0
K. pneumoniae	-	1	31	39	32	0.8
		2	33	32	38	1.2
		3	23	27	25	0.9
K. pneumoniae (non-mucoid)	-	1	61	40	58	1.5
		2	40	36	48	1.3
		3	41	35	38	1.1
K. oxytoca	-	1	52	47	45	0.9
		2	35	37	45	1.2
		3	35	40	41	1.0

* Average 3 replicates

TABLE 3. DETECTION OF *KLEBSIELLA* PERSISTING IN LOW NUTRIENT WATER BY m-KLEB AGAR

STORAGE TIME (DAYS)	FECAL *KLEBSIELLA** (PER mL)			NON-FECAL *KLEBSIELLA* (PER mL)		
	PLATE COUNT AGAR	m-KLEB AGAR	m-KLEB/PCA	PLATE COUNT AGAR	m-KLEB AGAR	m-KLEB/PCA
0	27,000	28,000	1.04	29,000	28,000	0.97
4	28,000	28,000	1.00	31,000	33,000	1.06
12	ND	54,000	-	ND	70,000	-
20	90,000	83,000	0.92	60,000	70,000	1.17

*FECAL COLIFORM POSITIVE AT 44.5°C.

TABLE 4. EVALUATION OF RESULTS WITH THREE KLEBSIELLA MEDIA ON VARIOUS NATURAL WATERS

WATER SOURCE	HETEROTROPHIC BACTERIAL COUNT PER mL	TOTAL COLIFORMS PER 100 mL	FECAL COLIFORMS PER 100 mL	KLEBSIELLA PER 100 mL				MEDIA	RATIO
				m-KLEB COUNT	m-KLEB VERIFIED	MCIC COUNT	M-K COUNT	m-KLEB / MCIC	m-KLEB / M-K
SPRING									
CLOUGH	20	39	2	5	4.5	1	2	4.5	2.3
LAKE									
BURNET	800	740	330	67	64	40	92	1.6	0.7
CREEKS									
MILL	190,000	32,000	2,200	9,300	8,700	7,300	3,300	1.2	2.6
SYCAMORE	470	170,000	8,300	33,000	33,000	30,000	36,000	1.1	0.7
RIVER									
PUBLIC LANDING	13,000	9,700	1,300	2,100	2,100	1,900	1,800	1.1	1.2
DOMESTIC SEWAGE									
SYCAMORE PRIM. EFF.	3,800,000	19,000,000	2,000,000	9,700,000	9,200,000	10,300,000	9,400,000	0.9	1.0

TABLE 5. DIFFERENTIAL CHARACTERISTICS OF WATERBORNE ORGANISMS ON m-KLEB AGAR*

SPECIES	TOTAL STRAINS	PERCENT DIFFERENTIATED			
		BLUE	PINK	YELLOW	CREAM
KLEBSIELLA PNEUMONIAE	43	97.7	2.3	0	0
KLEBSIELLA OXYTOCA	31	90.3	6.5	3.2	0
ENTEROBACTER CLOACAE	2	0	100.0	0	0
ENTEROBACTER AEROGENES	1	100.0	0	0	0
ENTEROBACTER AGGLOMERANS	3	33.3	66.7	0	0
CITROBACTER FREUNDII	2	0	100.0	0	0
ESCHERICHIA COLI	2	0	100.0	0	0
AEROMONAS HYDROPHILA	20	0	50.0	25.0	25.0
PSEUDOMONAS AERUGINOSA	1	0	0	0	0
PROTEUS VULGARIS	1	0	0	0	0
SERRATIA MARCESCENS	1	0	0	0	0
YERSINIA ENTEROCOLITICUS	1	0	0	100.0	0
VIBRIO SPP.	1	0	100.0	0	0
KLUGVERA SPP.	1	0	100.0	0	0

*ORGANISMS IDENTIFIED FROM 6 NATURAL SAMPLES EXAMINED BY m-KLEB AGAR.

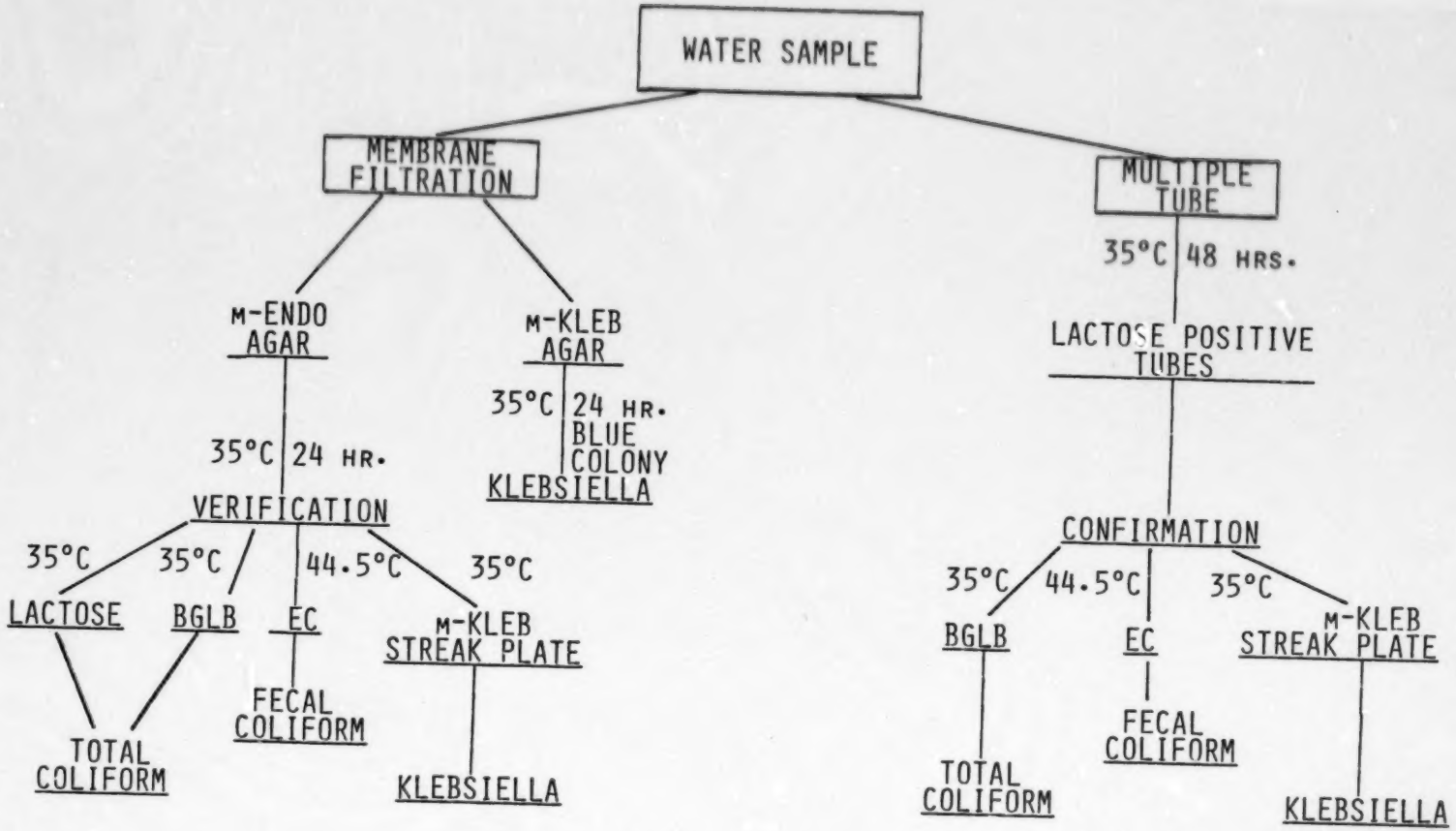

FIGURE 1. PATHWAYS FOR KLEBSIELLA DETECTION IN WATER SUPPLY